Bacterial inoculation manipulates the coral epigenome

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Keywords: climate change, pathogens, coral probiotics, Beneficial Microorganisms for Corals (BMCs), microbiome manipulation, DNA methylation

Summary

Environmental shifts can elicit epigenetic modifications in corals, which regulate gene expression and physiology, though it remains unclear if associated bacteria can also induce such changes. Here, we inoculated nubbins of the coral *Pocillopora verrucosa* with an opportunistic pathogen, *Vibrio coralliilyticus*, and/or a coral probiotic, *Cobetia* sp., and subjected the nubbins to heat stress. We discovered that the epigenomes responded already after 19 days to heat stress and 28 days to bacterial inoculation. Pathogen stress led to DNA methylation changes not seen in other treatments. Notably, the coral probiotic was able to mitigate specific epigenetic changes in a glutamate receptor pathway, and this correlated with increased stress resilience and higher survival rates of the corals. Importantly, epigenetic marks persisted even after the temperature stress was removed, suggesting epigenome acclimation. Thus, bacterial-induced changes to the coral epigenome may instigate long-term changes in host resilience.

Main

Recent work has shown that beneficial bacteria (probiotics) can increase coral health and resilience in response to stress caused by pathogens, increased temperature, and oil spills[1-7]. This is because corals are holobionts that rely on their associated symbiotic microbes for proper functioning and resilience[8-10]. For example, inoculation of the coral *Pocillopora damicornis* with beneficial microorganisms for corals (BMCs) consisting of bacteria from the genera *Yangia*, *Roseobacter*, *Phytobacter*, and *Salinicola*, correlated with increases in energy reserves and calcification rates[11]. Furthermore, administration of BMCs during and after heat stress increased transcription of genes involved in recovery and stress attenuation and decreased those related to inflammatory and innate immune responses in the coral *Mussismilia hispida*[5]. These changes also correlated with improved coral survival and increased photosynthetic efficiency in the associated symbiotic microalgae (i.e., Symbiodiniaceae)[5]. High temperatures, though, can also lead to dysbiosis and increased establishment of opportunistic pathogens, causing coral bleaching and death[5,12]. For example, inoculation with the temperature-dependent coral pathogen *Vibrio coralliilyticus* brought about a reduction in the skeletal density and abnormal crystal morphology in *P. damicornis*[3]. This could be attenuated by inoculation with a BMC consortium composed of bacteria from the genera *Pseudoalteromonas*, *Cobetia*, and *Halomonas*[3], showing the protective capacity of BMCs against pathogens as well as temperature stress.

Coral-associated microorganisms have recently been posited to have the ability to alter coral epigenomes, thus modifying phenotypic responses[13]. The most commonly studied epigenome modification in corals is DNA methylation, which, depending on the location of the methylated bases, prevents transcriptional noise and increases fidelity[14-16]. The interaction between coral epigenomes and their symbionts was recently demonstrated by exchanging the Symbiodiniaceae strain in the coral *Montastraea cavernosa* and applying thermal stress, which together produced changes in DNA methylation[17]. Exchanging or manipulating bacterial symbionts may have similar impacts on the coral host. These impacts could be either indirect, by changing the nutritional status or altering metabolic capacities[10], or direct, by producing metabolites that act as cofactors for epigenome-modifying enzymes[18] or encoding proteins that are homologs of bacteria-produced proteins capable of inducing epigenomic changes in mammals[13].

Here, we sought to determine whether inoculation with bacteria leads to changes in the coral epigenome that align with improved resilience to thermal stress. To do this, we regularly inoculated *P. verrucosa* nubbins with either a BMC (*Cobetia* sp.; hereafter "*Cobetia*"), a temperature-dependent pathogen (*V. coralliilyticus* BAA-450; hereafter "*Vibrio*"), or both bacteria (hereafter "Both"), and subjected the corals to a temperature stress regime (from 27 ˚C to 33 ˚C), followed by a recovery period. Heat-stressed corals are hereafter denoted with "H_", whereas corals kept at ambient temperature throughout the experiment are denoted with "A_". The mesocosm experiment encompassed 28 days, with samples taken before any treatment was applied (T0 = day 0), at the peak of the heat stress (T1 = day 19), and after a recovery period (T2 = day 28). DNA methylation

differences at the gene level were analyzed and linked to increases in the abundance of the respective inocula and coral phenotypic responses, to show that bacterial inoculation, in combination with temperature stress, can modulate the coral epigenome. Through co-inoculation of a probiotic and pathogen, we could further show that indicators of stress seen in the pathogen-inoculated corals were reduced with the co-inoculated BMC. This pattern could be observed in all components of the holobiont (host phenotype, host epigenome, symbiont photosynthetic efficiency, and microbiome) and prospects probiotic therapy as prevention of epigenomic disturbances caused by pathogenic infection.

Probiotic inoculation increases coral resilience

Cobetia inoculation positively affected coral resilience to temperature stress during a recovery period after high-temperature exposure. The photosynthetic efficiency (*Fv*/*Fm* values measured by Diving-PAM II) was significantly lower in heat-stressed corals than in ambient temperature corals at T1 (*Wilcoxon Rank Sum*: $W = 256$, $p < 0.001$) and T2 (*Wilcoxon Rank Sum*: $W = 248$, $p < 0.001$) (Fig. 1A and Supplementary Table 1a). The *Fv/Fm* values were not significantly different between treatment groups in either the ambient temperature corals (*Kruskal-Wallis*: $X^2 = 4.87$, df = 3, $p = 0.182$) nor the heat-stressed corals (*Kruskal-Wallis*: $X^2 = 3.97$, df = 3, $p = 0.265$) at T2. However, similar to previous findings (Rosado et al., 2019), the combination of heat stress and *Vibrio* inoculation (H_*Vibrio*) resulted in significantly more paling (*Kruskal-Wallis*: $X^2 = 8.49$, df = 3, $p = 0.037$; *Dunn test*: $p = 0.043$; Fig. 1B) and tissue loss (*Kruskal-Wallis*: $X^2 = 10.08$, df = 3, $p = 0.018$; *Dunn test*: $p = 0.014$; Fig. 1C) when compared to the control group (H_Control) after the recovery period (T2) (Supplementary Table 1b). Notably,

both the color intensity and percentage of live tissue in all other treatments within the same temperature regime, including the co-inoculation treatment, were indistinguishable from each other (Fig. 1B-C). Although the *Vibrio*-inoculated coral nubbins were paler than the nubbins from the other experimental groups at T2 in the heat-stressed corals, the color intensities of the live tissue remaining on the coral nubbins were not significantly different (*Kruskal-Wallis*: X^2 = 7.48, df = 3, p = 0.058; Extended Data Fig. 1).

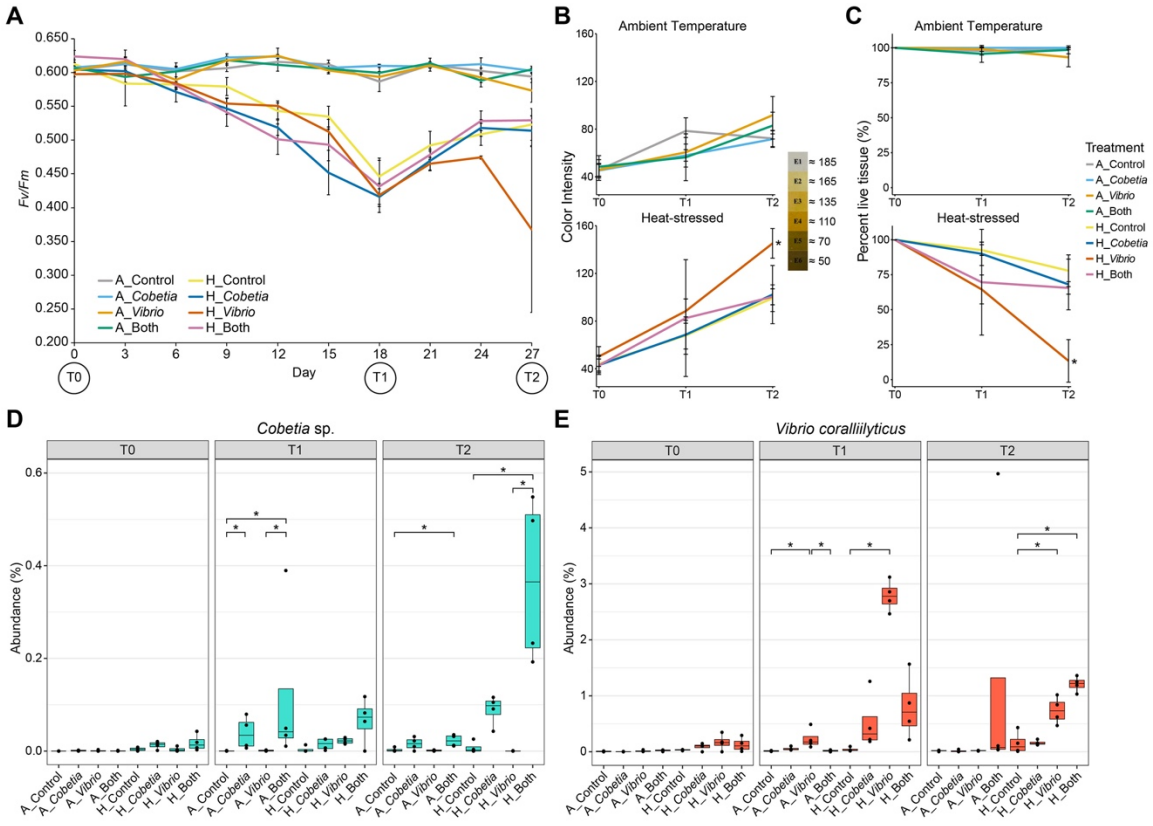


Fig. 1. Coral phenotypic response and inoculated bacteria abundances throughout the experiment. (A) Mean photosynthetic efficiency (*Fv/Fm* ratios; y axis) from coral nubbins during the mesocosm experiment days (x axis). The error bars represent standard deviations (n = 4). (B) Average color intensity and (C) percent live tissue of the coral nubbins in each condition, as measured by ImageJ. Lines represent the mean value for each condition, and the error bars represent standard deviations. T2 H_Vibrio corals were significantly different from the heat-stressed control (H_Control) corals in both color intensity and percent live tissue (*). Color intensity was measured on the CoralWatch Coral Health Chart[19] for reference. Percent abundances are shown in boxplots of the bacterial inoculums (*Cobetia* sp. (D) and *V.*

coralliilyticus (E)) across treatments and sampling times show significant differences between *Cobetia* sp. abundances at T1 and T2 and between *V. coralliilyticus* abundances at T1 and T2. Boxplots display the individual data points, medians, lower and upper quartiles, and whiskers displaying the 1.5x interquartile range. Abundances of each inoculum were compared between each treatment group (i.e., Control, *Cobetia*, *Vibrio*, Both) within each temperature condition (i.e., ambient temperature or heat-stressed). Asterisks denote significant differences between two groups within a timepoint ($p < 0.05$).

The differences in color intensity and tissue loss observed in the coral nubbins at T2 between the control group and the *Vibrio* treatment in the heat-stressed corals may be due to the establishment of the pathogen in the heat-stressed samples at T1 (Fig. 1D-E and Supplementary Table 2). Using 16S rRNA gene analysis, we found that the relative abundance of *V. coralliilyticus* was significantly higher in the corals treated with the pathogen than the control treatment at T1 (*Kruskal-Wallis*: $X^2 = 13.059$, df = 3, $p = 0.005$; *Dunn test*: $p = 0.002$) and T2 (*Kruskal-Wallis*: $X^2 = 11.647$, df = 3, $p = 0.009$; *Dunn test*: $p = 0.045$). Interestingly, the *V. coralliilyticus* abundance was lower at T2 than T1, which may be a sign of successional changes in the microbiome coinciding with disease progression[20], although data in this area is lacking. Overall, the co-inoculation with the BMC was able to mitigate *Vibrio* establishment in the microbiome during the peak of stress at T1, and controlled the relative abundance *Vibrio* to similar degree at T2. Moreover, the co-inoculation had a positive impact on the phenotype in heat-stressed corals at T2, contributing to the increased stress resilience and survival observed in this treatment.

The protective capacity of coral-associated bacteria, and probiotics in particular, to pathogen stress has been recently demonstrated by a single bacterium, *Pseudoalteromonas* sp. strain McH1-7, stopping or slowing the progression of stony

coral tissue loss disease in *Montastraea cavernosa* fragments[7]. The authors hypothesized that the disease suppression was due to antibacterial compounds produced by the bacterium[7]. Likewise, a single bacterial strain, *Phaeobacter* sp. BS52, was able to protect the seaweed *Delisea pulchra* from a pathogen-induced bleaching disease[21]. Although *Phaeobacter* sp. BS52 was found to be antagonistic towards pathogens, its ability to prevent dysbiosis was determined to be a main factor in blocking disease progression[21]. In both of these recent examples, the protective capacity of the probiotic was attributed to its interactions with other bacteria, independent of interactions with the host. In this experiment, microbiome differences were also observed between temperature groups, as well as bacteria treatment groups within each temperature condition (Extended Data Fig. 2 and 3, Supplementary Table 3a and 3b), which supports the concept that attenuation of pathogen-derived dysbiosis boosts coral resilience.

Coral epigenomes respond quickly to temperature stress and bacterial inoculation

To explore whether the stress mitigation in co-inoculated coral nubbins was also attributed to interactions with the coral epigenome, genome-wide differences in DNA methylation were analyzed in response to temperature stress, bacterial inoculation, and their interaction. The profile of median methylation levels of genes across the genome of *P. verrucosa* were plotted on non-metric multidimensional scaling (nMDS) plots at each timepoint, and the significance of each factor was determined (Fig. 2, Supplementary Table 4a). Significant differences existed at the peak of stress (T1) between corals exposed to the heat stress and those that were not (*PERMANOVA*: $R^2 = 0.107$, $F = 3.245$, $p = 0.001$; *PERMDISP*: $p = 0.981$), showing that methylation marks can change after

twelve days of constant exposure to temperature stress (and 19 days of experimentation). This is noteworthy, as although epigenome modifications, and DNA methylation specifically, have been shown to be reactive to environmental changes[15,17,22-25], correlate with phenotypic acclimatization[15,25], and be heritable[26], it is not known how quickly the epigenome can react to these changes. Plasticity of DNA methylation in corals has been tested on the scale of weeks[25,27] and months[17,23,24,28] to a year or more[14,15,22,28], with the shortest timescale in which DNA methylation changes has been observed being 40 days[25]. Thus, the findings here, that 12 days of heat stress can induce DNA methylation changes across the genome, represent a novel benchmark in coral epigenome research.

The difference between samples subjected to different temperature conditions was also observed after 9 days of recovery (28 days total; T2) (*PERMANOVA*: R^2 = 0.061, F = 1.955, p = 0.002; *PERMDISP*: p = 0.399). Additionally, timepoint was a significant factor in separating the heat-stressed corals using genome-wide DNA methylation levels, and pairwise comparisons showed significant differences between each of T0-T1, T1-T2, and T0-T2 (Extended Data Fig. 4). Bacteria inoculation treatments also significantly contributed to the separation of samples at T2 (*PERMANOVA*: R^2 = 0.112, F = 1.197, p = 0.028; *PERMDISP*: p = 0.412), but there was not statistical support for the interaction between temperature and bacteria treatments (*PERMANOVA*: R^2 = 0.106, F = 1.133, p = 0.106). Pairwise comparisons showed that pathogen-inoculated corals were significantly different from the control corals (*PERMANOVA*: R^2 = 0.101, F = 1.456, p = 0.029), but there was no pairwise significance between any of the other treatments at T2 (Supplementary Table 4b). The effects of bacterial inoculation on coral DNA methylation

patterns after 28 days and the continued difference in these patterns due to heat stress after 19 days and 28 days exemplifies persistence of epigenome modifications brought about by environmental change. Furthermore, these observations demonstrate that DNA methylation patterns can directly respond to active manipulation, which remains distinct from initial epigenetic marks even after temperature stress is removed.

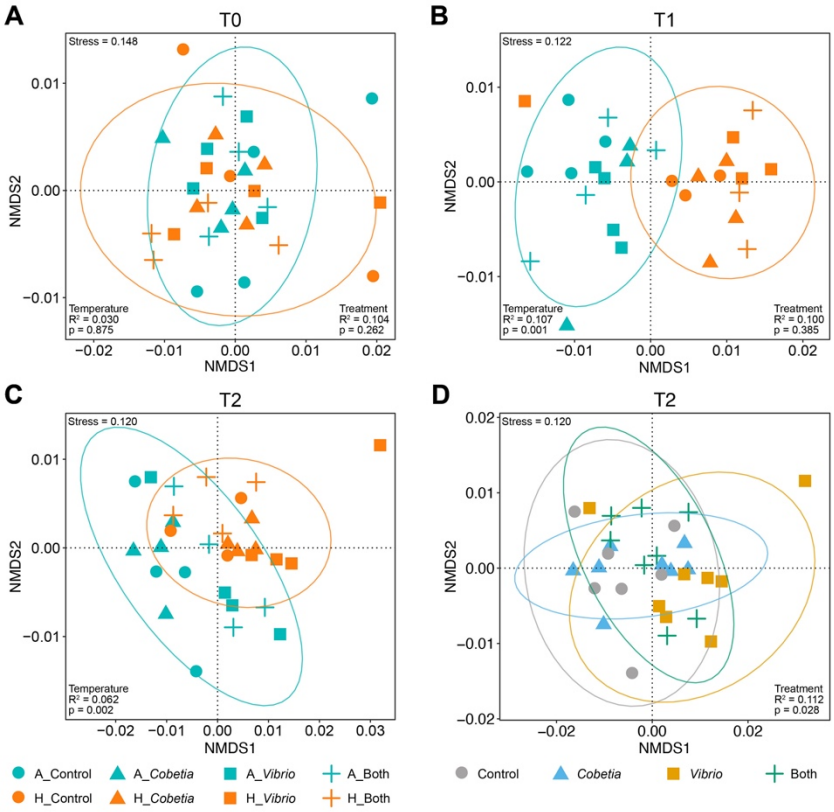


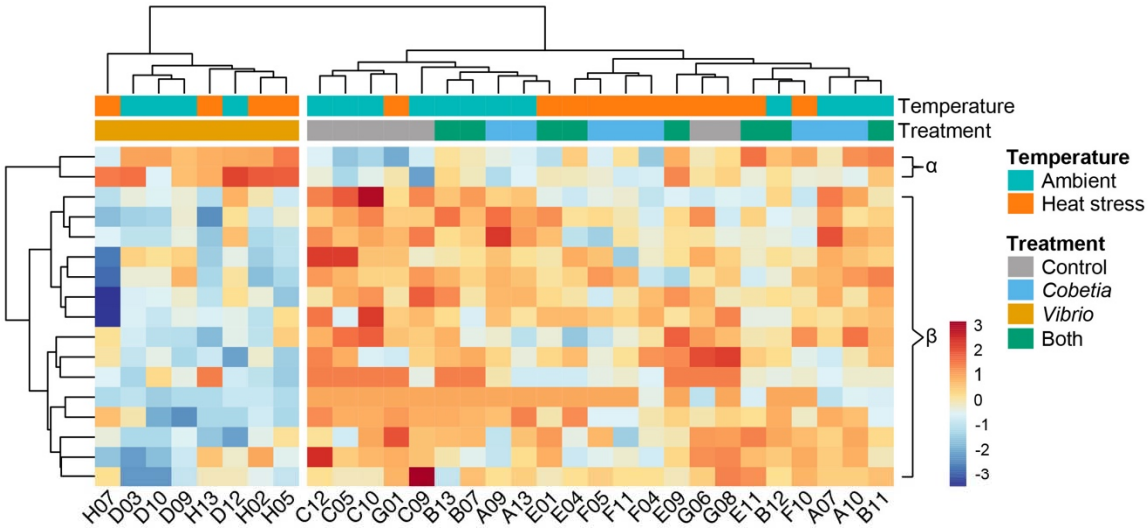
Fig. 2. Non-metric MultiDimensional Scaling (nMDS) plots based on Bray-Curtis dissimilarities of the median methylation levels of genes across the genome of *P. verrucosa*. There was no significant difference between temperatures and treatments at T0. (B) There was a significant difference between the ambient temperature treatments (blue) and the high temperature treatments (orange) at (B) the peak of stress (T1) and (C) after recovery (T2). (D) There was also a significant effect of treatment at T2. Pairwise comparisons revealed that the Control group and *Vibrio* group were significantly different. Circles = 95% confidence intervals around temperature treatments.

Recently, the concept of "epigenetic memory", where intragenerational differences between epigenomes of different individuals lead to vertical transmission of distinct epigenomes, was confirmed in the brain coral *Platygyra daedalea*[26]. This concept, however, has been recognized in plants for many years (reviewed in [29]). Here, the changes in epigenomes driven by environmental differences, as well as bacterial inoculations, signify the potential for long-term persistence after removal from environmental stress and show promise for microbiome manipulation experiments with implications for heritable variation in coral resilience.

Pathogen inoculation leads to changes in gene methylation

The significant genome-wide differences in median methylation levels observed between the temperature conditions at T1 and the temperature conditions and bacteria treatment groups at T2 (Fig. 2) were further investigated using pairwise generalized linear models (GLMs). A total of 932 genes were significantly associated with temperature differences at T1, and 313 genes were significantly associated with the different temperature groups at T2 (Extended Data Fig. 5, Supplementary Table 5a and 5b). At T2, 17 genes were found to be significantly influenced by *Vibrio* treatment in comparison to the control group, 15 of which had depleted median methylation levels correlated with *Vibrio* inoculation (Supplementary Table 5c). Extending the comparison of these 17 genes to the other treatment groups using hierarchical clustering based on Euclidean distances shows that coral nubbins inoculated with *Vibrio* form an outgroup from the other three conditions at T2 (Fig. 3). These results corroborate the stressed physiological response of

the coral nubbins caused by *Vibrio* inoculation observed at T2 (Fig. 1B-C). This also demonstrates that, although pathogen inoculation significantly alters coral DNA methylation, co-inoculation of the pathogen and BMC leads to methylation patterns similar to the control group, evidencing the BMC's ability to negate impacts caused by pathogen stress on coral health.



GO:ID	Enriched biological process found in cluster β
GO:0072673	lamellipodium morphogenesis
GO:0034498	early endosome to Golgi transport
GO:0035871	protein K11-linked deubiquitination
GO:0035627	ceramide transport
GO:0035520	monoubiquitinated protein deubiquitination
GO:0007196	adenylate cyclase-inhibiting G protein-coupled glutamate receptor signaling pathway
GO:0070212	protein poly-ADP-ribosylation
GO:0007097	nuclear migration
GO:0002181	cytoplasmic translation
GO:0036297	interstrand cross-link repair
GO:0008053	mitochondrial fusion
GO:0006282	regulation of DNA repair
GO:0035082	axoneme assembly
GO:0051966	regulation of synaptic transmission, glutamatergic

Fig. 3. Genes with median methylation levels significantly associated with *Vibrio* treatment according to GLMs comparing samples from treatment groups at T2. There is a separation between the corals inoculated with *V. coralliilyticus* and the other treatments (x-axis). Hierarchical clustering was performed using Euclidean distances and the "ward.D2" agglomeration method. Colors represent the z-score for any given gene. The enriched GO terms using TopGO display the significant functions with depleted methylation levels in pathogen-stressed corals (cluster β). All enriched GO terms are in Supplementary Table 6c.

Increases in gene body methylation reduce spurious transcription in highly expressed genes, thereby reinforcing the epigenetic control of genes as expression increases[14-16]. Our findings here revealed that methylation within *P. verrucosa* occurred primarily within the gene body (Extended Data Fig. 6, Supplementary Table 9). Genes with depleted methylation in the corals treated with *Vibrio* only (cluster β in Fig. 3) highlight the link between pathogen stress and diminished epigenetic control of genes involved in cell maintenance and functioning and genes related to the immune system, which potentially correlates with decreased gene expression.

The GO terms “protein K11-linked deubiquitination”, “monoubiquitinated protein deubiquitination”, “adenylate cyclase-inhibiting G protein-coupled glutamate receptor signaling pathway”, “cytoplasmic translation”, “axoneme assembly”, and “regulation of synaptic transmission (glutamatergic)” all function in the metabolic and cellular processes of the coral cell. Both the “adenylate cyclase-inhibiting G protein-coupled glutamate receptor signaling pathway” and the “regulation of synaptic transmission (glutamatergic)” functions rely on the neurotransmitter glutamate. Glutamate production is necessary for algal symbiosis in the cnidarian model *Aiptasia*[30]. Moreover, in the coral *Stylophora pistillata*, heat stress was found to disrupt glutamate biosynthesis and trigger glutamate catabolism, which contributed to coral bleaching[31]. Therefore, pathogen inoculation may exacerbate losses in glutamate caused by heat stress by reducing the epigenetic control of glutamatergic signal transduction. This may be further supported by significant differences at T2 in the GO term “glutamatergic synapse” (GO:0098978) due to temperature conditions (Supplementary Table 6b), although additional research in this

area is necessary, as heat stress did not induce uniform methylation trends in all of the affected glutamatergic synapse genes (Extended Data Fig. 7).

Another group of genes, which have roles in the coral immune system, showed depleted median methylation levels due to *Vibrio* inoculation treatment. These include genes defined as “lamellipodium morphogenesis”, “early endosome to Golgi transport”, and the well-known stress response mechanisms “interstrand cross-link repair” and “regulation of DNA repair”. Lamellipodia, and by association “lamellipodium morphogenesis”, control interactions with the algal symbiont Symbiodiniaceae[32] and contribute to wound healing in vertebrates and invertebrates, which has been demonstrated in the cnidarian *Clytia hemisphaerica*[33]. The reduction in DNA methylation of “early endosome to Golgi transport” is also consistent with previous research, as endosomal pathways are targeted by *Vibrio* during infection to evade host defenses and increase the survival of the bacterium[34]. Specifically, toxins produced by *V. coralliilyticus* cause the disruption of endosome development and downregulation of immune genes in the host[34,35].

Together, this suggests a depletion in the methylation of genes related to metabolic and cellular processes and immune responses in pathogen-stressed corals, which could be a result of downregulation of specific genes observed to be affected by *Vibrio* virulence[36]. Alternatively, pore forming toxins may both modulate epigenetic machinery and disrupt host signaling pathways, as has been documented in humans[37]. The altered methylation patterns in *Vibrio*-inoculated corals further indicate the ability of *Vibrio* to manipulate the coral host defense genes, and the lack of difference in methylation patterns between co-

inoculated corals and the other treatments show the probiotic's ability to mitigate the *Vibrio*'s effect on the coral epigenome.

Gene methylation differences are correlated with phenotypic response

To determine which genes affected by *Vibrio* inoculation (Fig. 3) correlated most with the paling and tissue loss observed in the corals (Fig. 1), linear models were implemented using the median methylation levels and color intensity as factors (Supplementary Table 7). The genes showing the highest correlation between methylation percentage and phenotypic response ($R^2 > 0.100$ and $p < 0.05$) were g16333, g1533, g17345 , g14350, and g8986 (Fig. 4). The GO terms describing these genes were "negative regulation of transcription by RNA polymerase II" (GO:0000122), N/A, "ceramide transport" (GO:0035627), "adenylate cyclase-inhibiting G protein-coupled glutamate receptor signaling pathway" (GO:0007196), and "cilium movement" (GO:0003341), respectively. Of the genes with defined functions, three are related to cellular processes, while g17345 ("ceramide transport") functions in macromolecule localization within the cell. In all of the defined genes, *Vibrio*-inoculation led to a significant depletion in DNA methylation when compared to the control group in either the ambient temperature or heat-stressed conditions. Interestingly, in all but one gene (g16333), co-inoculation with *Cobetia* prevented similar decreases in DNA methylation when compared with the control group.

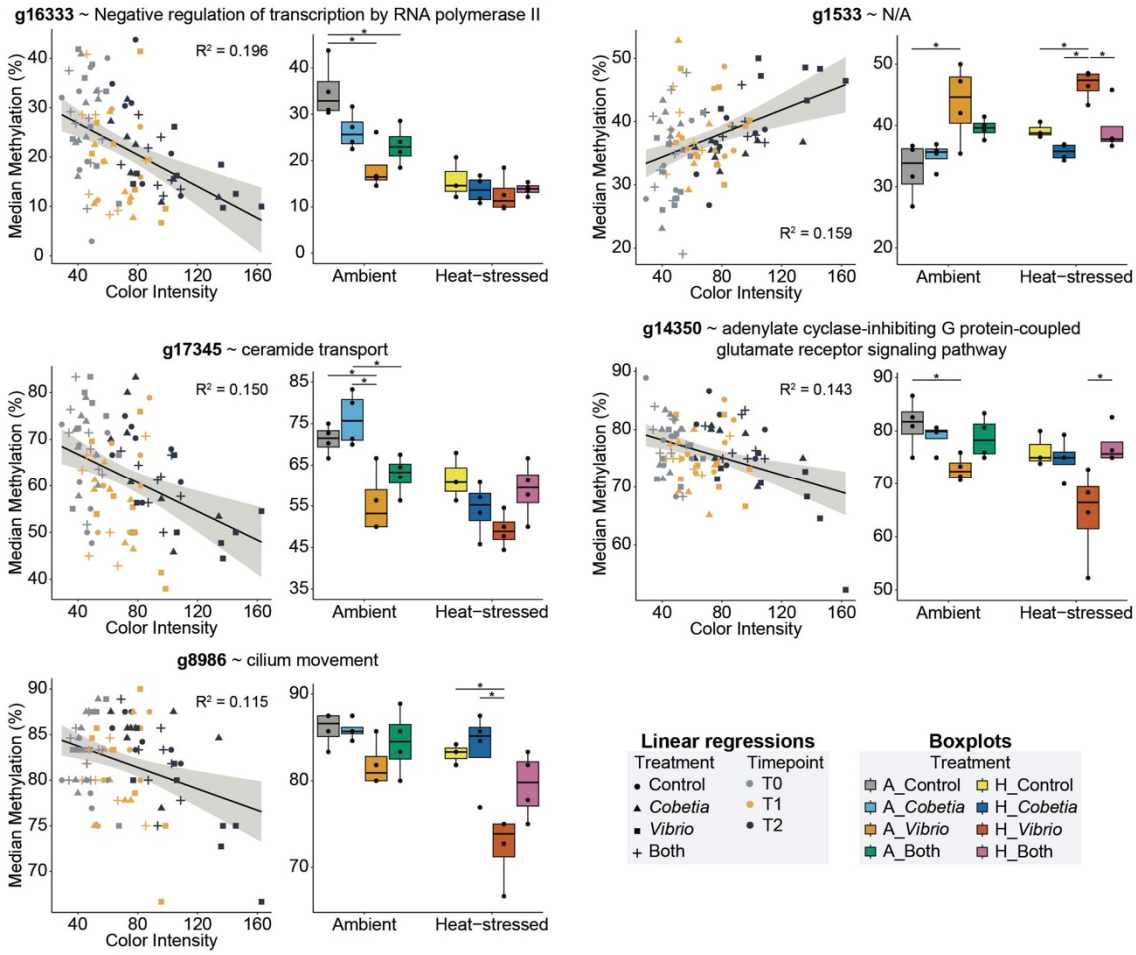


Fig. 4. Methylation profiles of genes with significant correlations ($R^2 > 0.100$, $p < 0.05$) between methylation levels and phenotypic response (coral nubbin color intensity). Linear regression plots show the correlation lines of the coral nubbin color intensity to the median methylation percent in each sample along with the 95% confidence intervals (shaded regions). Boxplots showing the median methylation percent of the samples at T2 (after recovery), were used to compare between treatments with one-way ANOVA, followed by TukeyHSD. Asterisks denote significance between treatment groups in the boxplots ($p < 0.05$). Boxplots display the individual data points, medians, lower and upper quartiles, and whiskers displaying the 1.5x interquartile range. The effect of *Vibrio*-inoculation was mitigated in the co-inoculated corals in the heat-stressed conditions in three of these genes.

The gene g14350, defined by the GO term "adenylate cyclase-inhibiting G protein-coupled glutamate receptor signaling pathway", had significantly lower median methylation levels in *Vibrio*-treated corals when compared with the control group in the

ambient temperature conditions and the co-inoculation group in the heat-stressed conditions. As discussed above, the neurotransmitter glutamate mediates interactions between corals and their algal symbionts[30]. Interestingly, glutamate receptor inhibition leads to disruption of this association[38]. Thus, the decrease in epigenetic control over this glutamate receptor pathway due to pathogen stress may similarly interfere with algal symbiosis, which correlates with diminished coral health.

When analyzing the methylation levels of the genes and putative functions, we can conclude that *Vibrio* inoculation caused changes to methylation levels in metabolic and cellular processes, which corresponded with phenotypic responses (Fig. 1). The difference between the *Vibrio*-inoculated corals and the other three conditions (control, inoculation with *Cobetia* sp., and the co-inoculation) in these genes at T2 demonstrates the coral probiotic's ability to prevent changes to the coral epigenome in key functions altered by the pathogen (*V. coralliilyticus*). This effect was associated with increased resilience to stress and higher survival rates.

The direct mechanisms by which bacteria are able to elicit changes to the coral epigenome are still to be explored, although metabolites and proteins produced by coral-associated bacteria are suggested to play a role[13,18]. Additionally, the crosstalk between coral epigenomes and coral-associated bacteria may not be unidirectional, as pathogen susceptibility has been hypothesized to be at least partly controlled by epigenetic factors in aquaculture fish[39]. By connecting rapid microbiome changes to more long-term and heritable epigenetic changes (Fig. 5), bacteria inoculation has the potential to induce lasting differences, even without direct establishment in the bacterial community. Thus,

as future studies explore this interaction further, the current paradigm that microbiome interventions only lead to short-term and volatile changes[40] might shift.

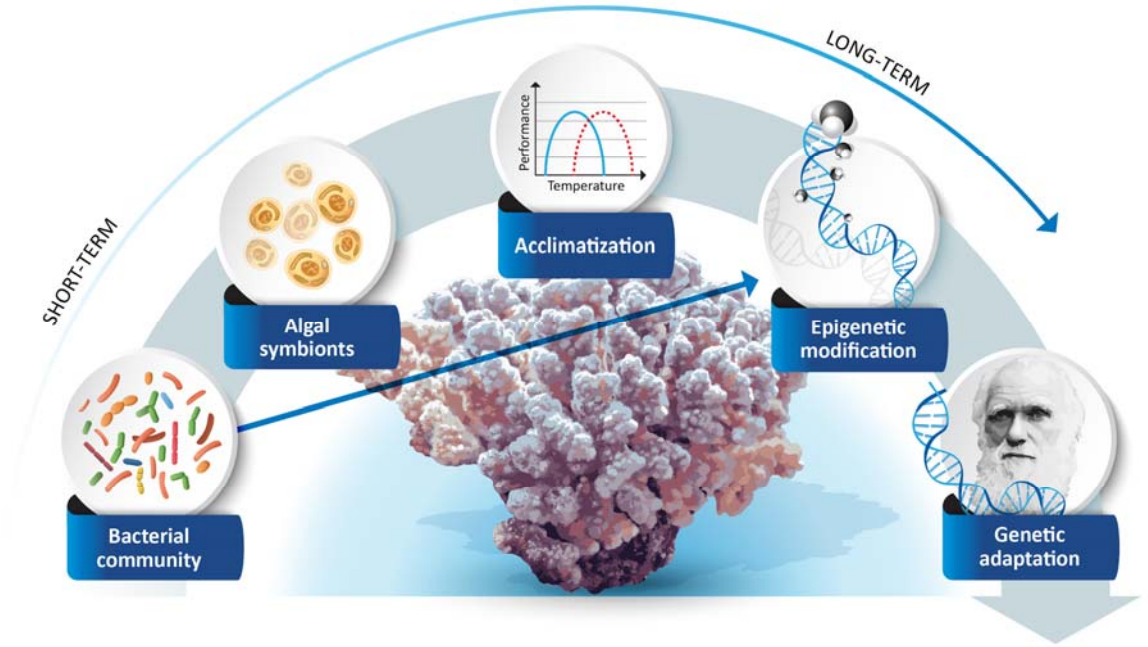


Fig. 5. Rethinking the flexibility and longevity of coral microbiomes via crosstalk with coral epigenomes. Microbiome changes occur quickly and are fleeting. However, relating microbiome manipulation and coral DNA methylation here shows that bacterial community changes may induce long-term and possibly heritable changes in corals by influencing modifications to the coral epigenome. This figure highlights the transformative nature of our results by adding an "arrow" to the previous concept postulated in ref.[41], connecting the bacterial community with epigenetic modification.

Conclusions

Although hypothesized, the interaction between changes in the coral-associated microbiome and the coral epigenome was yet to be demonstrated[13,18]. The findings of this study are fourfold, as we show that, in corals, (1) alterations in DNA methylation can readily be found after 19 days in different environmental conditions; (2) bacterial inoculation, as well as temperature stress, contribute to differences in DNA methylation; (3) pathogen inoculation causes changes in DNA methylation to specific cellular

processes and immune responses; and (4) a single coral probiotic (*Cobetia* sp.) can mitigate changes in DNA methylation of specific genes and the phenotypic response induced by pathogen inoculation (with *V. coralliilyticus*). These results have the potential to influence how customized microbiome rehabilitation experiments[42,43] is utilized for increasing resistance and resilience in vulnerable corals.

Methods

Coral collection and experimental setup

In order to prevent intergenomic variability would obfuscate identification of methylation marks[44], clonal fragments from *Pocillopora verrucosa* were collected from the Al Fahal reef in the central Red Sea (22˚ 18.3071’ N, 38˚ 57.8741’ E) on 14 March 2022 (*in situ* seawater temperature = 25˚C) and brought to the aquariums at the Coastal and Marine Resources Core Lab (CMR) in King Abdullah University of Science and Technology (KAUST). All coral husbandry and experimentation was done under the supervision of KAUST's Institutional Biosafety and Bioethics Committee (IBEC). The coral colony was acclimated to CMR conditions (using a 300 L open-system aquarium tank, with a maximum intensity of 180 µmol of photons m−2 s−1 between 10:00 to 14:00, and a 12-hour day/night cycle changing at 7:00 and 19:00) and brought to 27˚C over the course of three weeks (approximately 0.5˚C increase every two days, followed by two weeks at 27˚C). Corals were further fragmented into finger-sized nubbins on 4 April 2022. After a five-week recovery period, nubbins were divided into the eight experimental tanks which housed the replicates for each treatment group (10 L aquarium tanks, using the same light parameters as the acclimation tank above) containing 0.22 µm filtered seawater. The coral nubbins were acclimated for seven days (until the average *Fv/Fm* ratio, a measure for photosynthetic efficiency and proxy for coral health, from the coral nubbins in each experimental tank was >0.600, using a Diving-PAM II; Walz, Germany).

The bacteria used in the experiment were the BMC *Cobetia* sp. (isolated from *Pocillopora verrucosa*[45]) and the opportunistic pathogen *Vibrio coralliilyticus* BAA450 (isolated from *Pocillopora damicornis*[46]). The bacteria cultures used in this experiment were grown using Marine Broth 2216 (Becton Dickinson, USA) for 24 hours at 27˚C before each inoculation. To minimize additional carbon input from the culture media, the cultures were spun at 6000 x g for 5 min and the bacteria pellets were resuspended in sterilized 3.5% NaCl solution. This process was repeated for a total of two washes, then the bacteria were resuspended in sterilized 3.5% NaCl solution for inoculation. Abundance and purity of each of the cultures were checked by the plate counting method using Marine Agar 2216 (Becton Dickinson, USA).

Starting on day 1, bacteria were inoculated into each of the treatment tanks every three days at a final concentration of 10^5 cells/mL. Thirty percent of the water was replaced with new 0.22 µm filtered seawater (kept at the same temperature of the experimental tanks) every other day. The experiment was performed over 28 days. Of the eight experimental tanks, four were maintained at 27˚C throughout the entirety of the experiment (denoted with an "A" for ambient temperature). In the other four experimental tanks, starting from day 1, temperature was increased from 27˚C to 33˚C (by increasing the temperature 1˚C per day for 6 days), then maintained at 33˚C for 12 days (until the average *Fv/Fm* across all conditions was <0.450), then returned to 27˚C (by decreasing the temperature 1˚C per day) where they were kept until the end of the experiment (4 days). These tanks are denoted with an "H" for heat stress. The four experimental tanks in each of A or H, contained one of the following experimental

conditions: corals with no inoculation ("Control"), corals inoculated with *Cobetia* sp. at a final concentration of 10^5 cells/mL ("*Cobetia*"), corals inoculated with *V. coralliilyticus* at a final concentration of 10^5 cells/mL ("*Vibrio*"), and corals inoculated with both *Cobetia* sp. and *V. coralliilyticus*, each at a final concentration of 10^5 cells/mL ("Both"). In total the eight conditions were: A_Control, A_*Cobetia*, A_*Vibrio*, A_Both, H_Control, H_*Cobetia*, H_*Vibrio*, and H_Both.

Samples of four coral nubbin replicates (n = 4) per tank were taken at the start of the experiment before any bacterial inoculation (T0), on day 19 (peak of stress, T1), and on day 28 (after recovery, T2). At each sampling time, the apical tip of the sampled nubbin was removed and flash frozen in liquid nitrogen for nucleic acid analysis. Frozen nubbins were then transferred to -80˚C until processing.

Coral health parameters

Coral health was monitored during the experiment using a Diving-PAM II (Walz, Germany) to measure the *Fv/Fm* ratios. *Fv/Fm* measurements were taken after dark acclimating the coral nubbins for at least 1 hour the night before each inoculation day (every three days starting on day 0) throughout the entirety of the experiment (n = 4 for each experimental tank). Images were taken of the coral nubbins at T0 and at their respective sampling times (T1 or T2).

ImageJ was used to analyze the percent live tissue, color intensity, and color intensity of live tissue of each of the images of the nubbins from sampling times T0 (n = 12 for each treatment), T1 (n = 4 for each treatment), and T2 (n = 4 for each treatment). For the percent live tissue, the area of live tissue on the coral nubbin was divided by the total area of the coral nubbin in each respective image. The color intensity was measured by first converting each image to grayscale and measuring the unweighted average in color intensity (from 0 = black, to 255 = white) incorporating the whole image of the coral nubbin. Thus, a lower average color intensity would represent healthier living tissue, and a higher color intensity would represent pale, bleached, or dead coral tissue. This, then, incorporates the color of the live tissue and any exposed coral skeleton that may be present in coral nubbins with tissue loss. For the color intensity of live tissue, only the unweighted average in color intensity observed in the live tissue portion of the image was considered. Percent live tissue, color intensity, and color intensity of live tissue measurements were compared between the samples with Kruskal-Wallis H tests[47], followed by Dunn's multiple comparison test[48] using Benjamini-Hochberg adjustment.

16S rRNA gene amplicon sequencing

DNA was extracted from airbrushed coral tissue using AllPrep DNA/RNA Mini Kit (Qiagen, Germany), following manufacturer's instructions. Extracted DNA was used for analysis of the bacterial community as well as identification of the methylation marks within the coral genome. For bacterial community analysis, the DNA composing the V3-V4 region of the 16S rRNA gene was sent to Novogene (Novogene, China) for

sequencing using a NovaSeq 6000 (Illumina, USA) with the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3').

Library preparation and sequencing of methylated DNA

Libraries of converted methylated DNA were prepared using the NEB Enzymatic Methyl-seq Kit (New England Biolabs Inc., USA) with the EM-seq Index Primers (New England Biolabs Inc., USA). Prepared libraries were sent to Novogene (Novogene, China) for sequencing using a NovaSeq 6000 (Illumina, USA). Due to insufficient DNA quality in 5 samples, 91 of the 96 samples were used for downstream analysis (n = 4 for each experimental group; except A_Control at T1; H_Control at T0, T1, and T2; and H_Both at T1; which all had n = 3).

Coral microbiome data analysis

For microbiome analysis, primer sequences were removed using cutadapt[49]. Sequences were further filtered and trimmed using the DADA2 pipeline[50]. Taxonomy was assigned using the SILVA_nr99_v138.1 database[51]. Sequences identified as Bacteria that did not match to Chloroplast or Mitochondria were further analyzed using phyloseq[52]. ASVs with a 100% match to the 16S rDNA sequence of the inoculums (*Cobetia* sp. or *Vibrio coralliilyticus*) via BLASTN[53] were determined to be representative ASVs of the inoculated bacteria. Statistical differences between relative abundance data in different groups were determined using the *vegan* package in R (version 4.3.1). Bacterial

abundances were not normally distributed (*Shapiro-Wilk*: $p < 0.05$), so significant differences between groups were determined by non-parametric tests. Kruskal Wallis H tests[47], followed by Dunn's multiple comparison tests[48], were used to find significant differences between treatment groups (n = 4 for each experimental group).

Microbiome composition differences were calculated between treatment groups in each of the temperature conditions and timepoints using principal coordinate analyses (PCoA). Significant factors driving the separation between samples and sample dispersion were determined using the adonis2 and betadisper commands in the *vegan* R package (version 4.3.1). Pairwise differences between treatment groups were tested using the pairwise.adonis command in the *pairwiseAdonis* R package.

Coral DNA methylation

DNA methylation was analyzed bioinformatically following the steps described in Liew et al. (2020)[26]. Reads were first trimmed using TrimGalore v0.6.10[49]. Then, the filtered reads were mapped to a reference genome for *Pocillopora verrucosa*[54] and deduplicated, before methylation marks were scored per-position using Bismark v0.23.1[55]. Methylated DNA positions were only considered bona fide if they met the criteria defined in Liew et al. (2020)[26]. Briefly, the probability of methylated positions resulting from sequencing error needed to be below 0.05 after correcting for false discovery rate, and the methylated reads were only kept if the methylation occurred in all replicates of at least one of the experimental conditions. Also, the median coverage needed to be ≥ 10 and minimum

coverage needed to be ≥ 5 across all of the samples in order to be considered for further analyses[26] (Supplementary Table 8).

The median methylation levels across genes were compared between groups. Each methylated position within a given gene was given a score between 0 (no methylation in any of the cells) to 1 (fully methylated across all cells), and the median across each gene was used for further analyses. Only genes with five or more methylated positions were used for downstream analyses. Nonmetric multidimensional scaling (nMDS) plots were used to compare the median methylation across the genome of the coral using the above cutoffs. Significant factors driving the separation between samples and sample dispersion were determined using the adonis2 and betadisper commands in the *vegan* R package (version 4.3.1). Pairwise differences between treatment groups were tested using the pairwise.adonis command in the *pairwiseAdonis* R package.

To extrapolate the genes related to the difference observed between the Control treatment and the *Vibrio*-treated corals at T2, generalized linear models (GLMs) were implemented in R using the following formula:

```
glm(median_methylation_levels ~ temperature + treatment, family = gaussian())
```

for the data from T2, where median methylation levels for each gene was compared with the predictor variables temperature (ambient vs. heat-stressed) and treatment (by using the Control treatment as a baseline to compare with each of *Cobetia*-treated, *Vibrio*-treated, and the co-inoculation treatment samples). Genes with fewer than 5 methylated positions were removed from the analysis. Genes with *Vibrio* treatment as a significant

predictor variable (after correcting for false discovery rate) were plotted on a heatmap with all of the T2 samples using hierarchical clustering with Euclidean distances and the "ward.D2" agglomeration method.

Functional enrichment of differently methylated genes

GO term annotations were collected from the published *P. verrucosa* genome[54]. GO term enrichment analysis was performed using TopGO[56] on the genes with significantly different methylation levels from the previous step. Enriched GO terms with $p < 0.05$ and were considered significant.

Correlating median methylation with phenotypic parameters

The genes predicted to be associated with *Vibrio* inoculation in the previous step were then tested against a phenotypic response variable using linear models with the formula:

```
lm(median_methylation_levels ~ color_intensity, data = gene)
```

to determine which of these genes correlated with phenotypic differences observed in the corals affected by temperature and treatment. The genes with $R^2 > 0.100$ and $p < 0.05$ were plotted using linear regressions with 95% confidence intervals. Boxplots showing the median methylation levels of these genes in each sample at T2 were then used to compare differences between groups using one-way ANOVA followed by TukeyHSD.

Data availability

EM-seq data is deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA1123499.

Acknowledgements

We would like to thank the Coastal and Marine Resources Lab and Bioscience Core Lab for their support and assistance throughout the experiment. This work was supported by KAUST grant number BAS/1/1095-01-01

Author contributions

A.R.B., H.D.M.V., G.C., T.T., M.A., C.R.V., and R.S.P. designed the experiments; A.R.B., H.D.M.V., P.M.C. conducted the mesocosm experiment and sample collection; A.R.B. and N.D. performed DNA extractions; A.R.B. and F.C.G. performed analyses; A.R.B. wrote the manuscript with input from all authors; A.S.R. and R.S.P. provided guidance and funding during the experiment.

Competing interests

The authors declare no conflicting interests.

Materials & Correspondence

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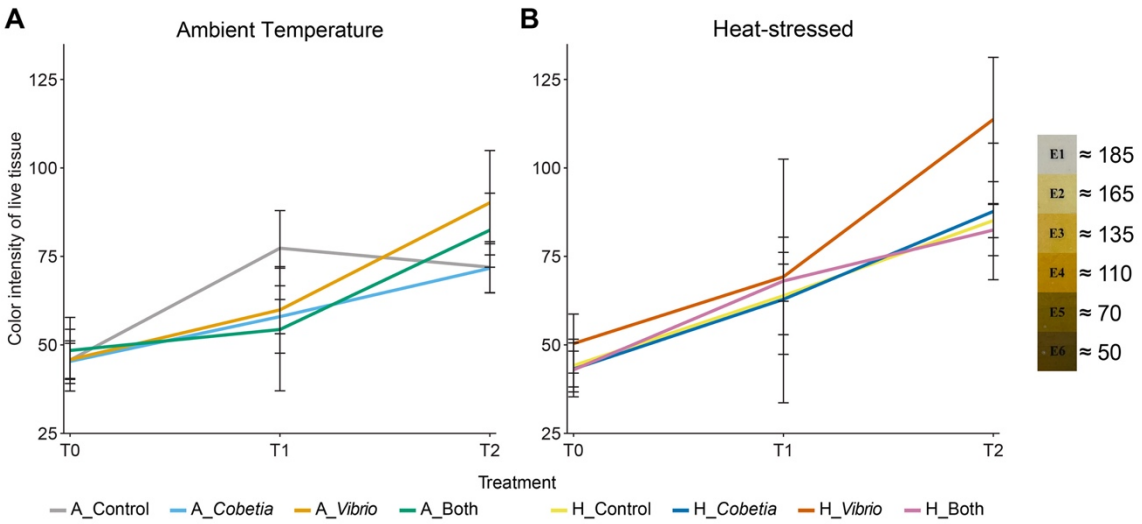
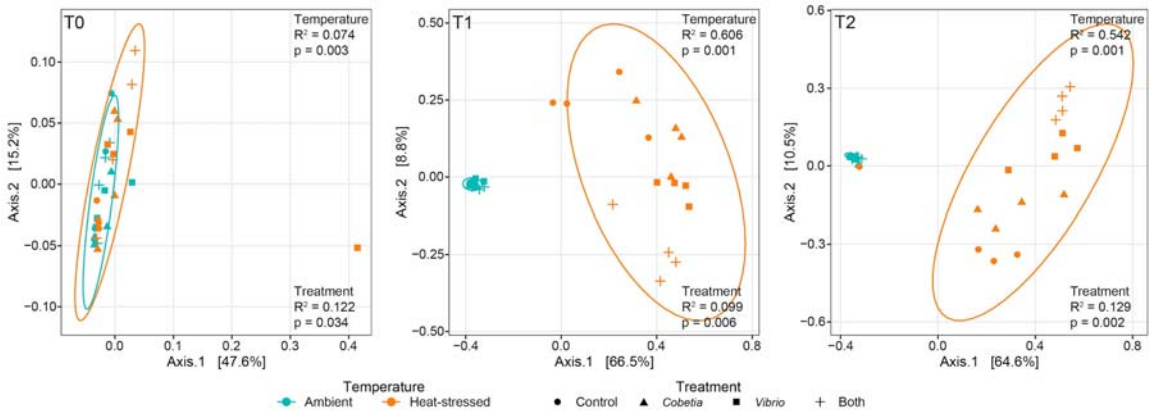
Supplementary Information

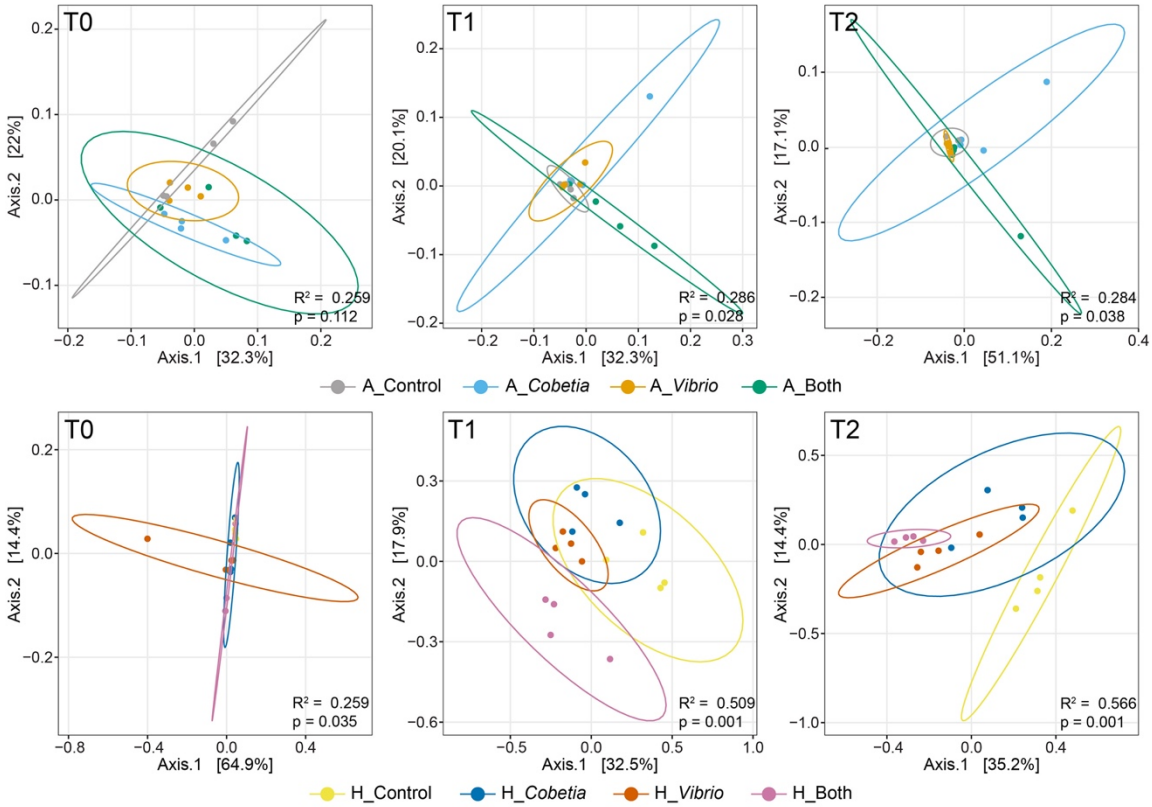
Supplementary tables are provided in excel format.



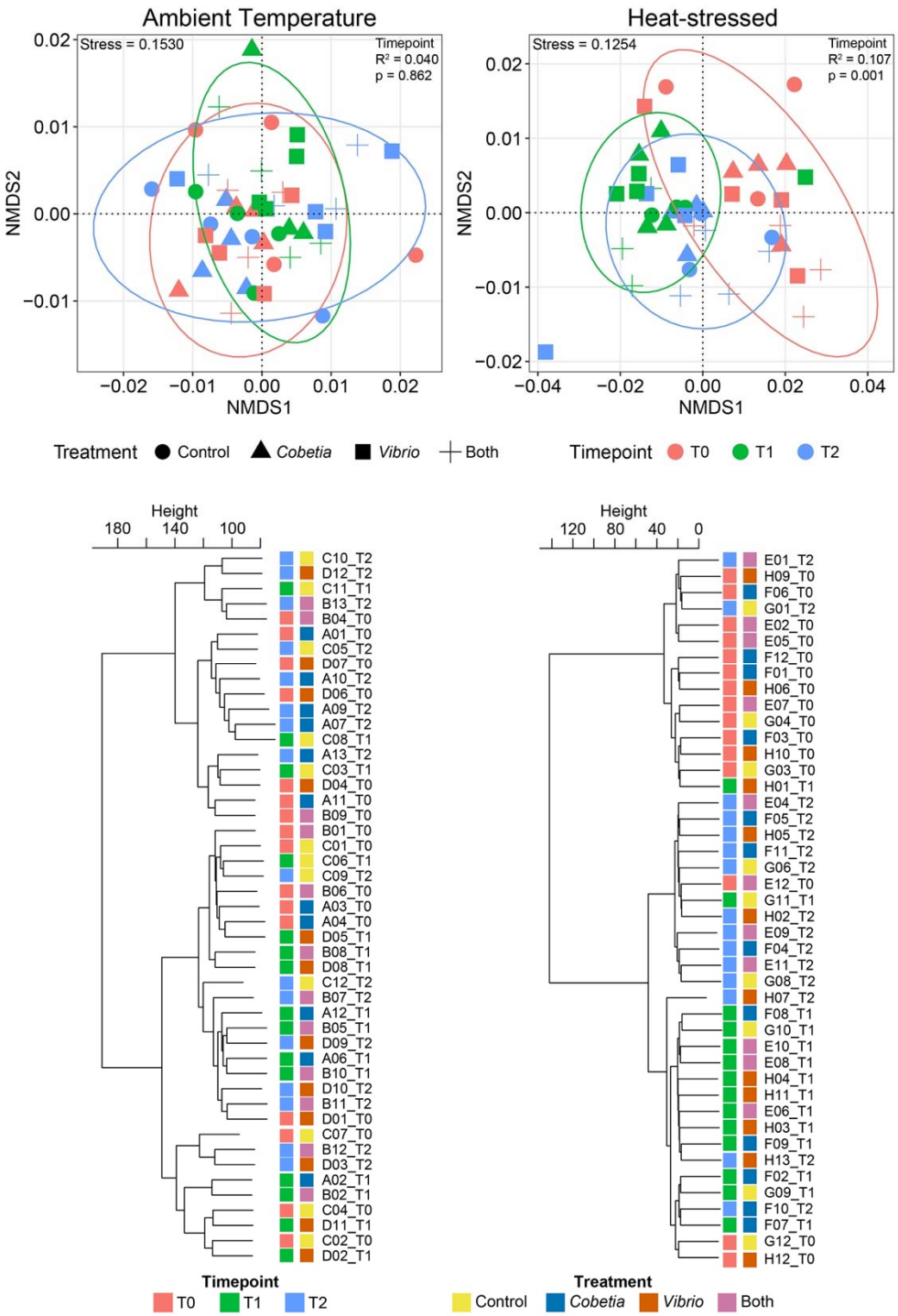
Extended Data Fig. 1. Color intensity of live tissue for the coral nubbins throughout the experiment. Lines represent the mean color intensities are plotted for each bacterial treatment group at each timepoint for nubbins in the (A) ambient temperature condition and (B) heat-stressed condition, as measured by ImageJ. The error bars represent standard deviations. Color intensity was measured on the CoralWatch Coral Health Chart[19] for reference.



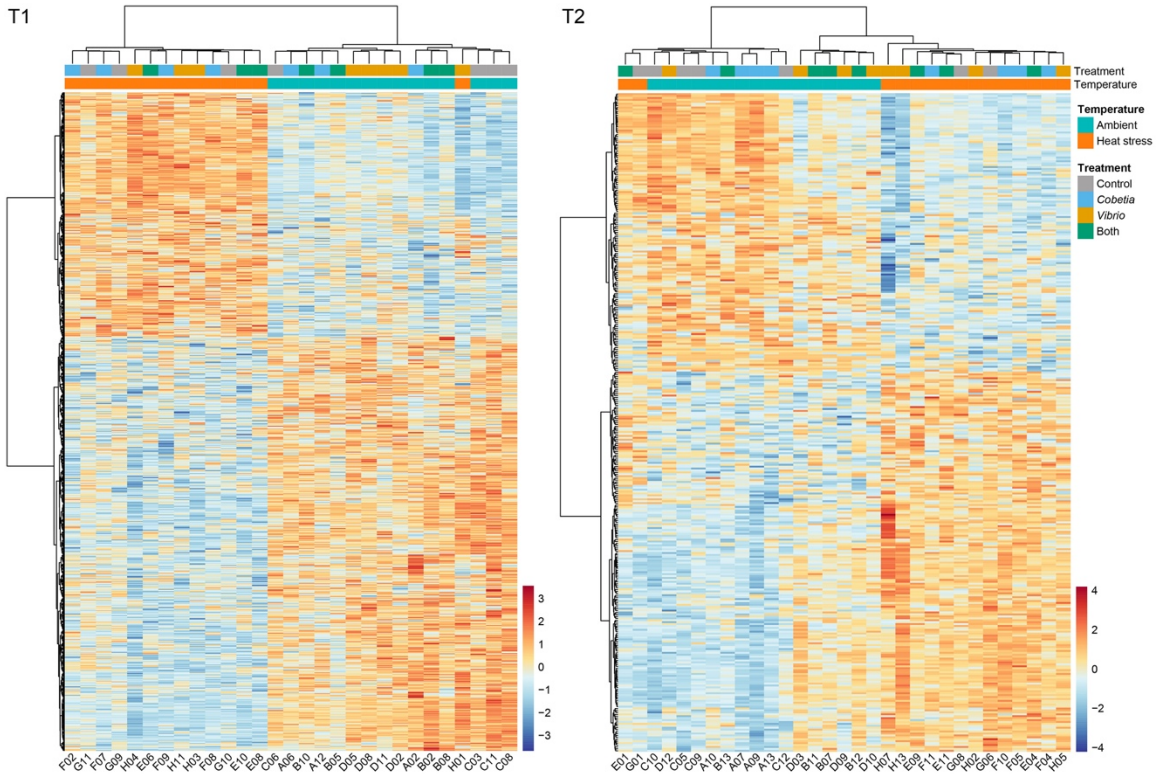
Extended Data Fig. 2. Principle coordinate analysis plots based on Bray-Curtis dissimilarities of the relative abundances of the total bacterial community in the coral nubbins at each sampling time. There were significant differences between temperature conditions and bacteria treatments at all timepoints. Sample dispersion was found to be significant between temperature conditions at T1 (*PERMDISP*: $p < 0.001$) and T2 (*PERMDISP*: $p < 0.001$). Circles = 95% confidence intervals around temperature conditions.



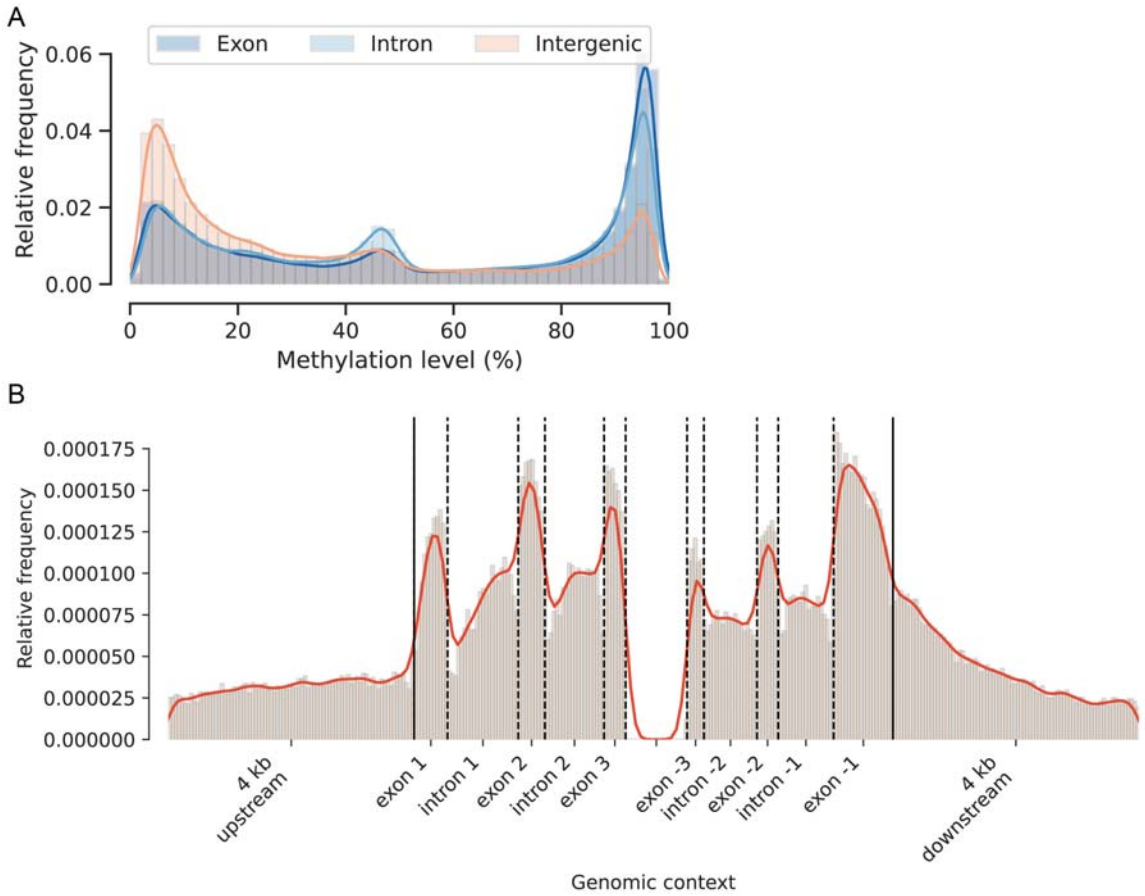
Extended Data Fig. 3. Principal coordinate analysis (PCoA) plots of microbiome data showing the separation between treatments using Bray-Curtis dissimilarity between samples in ambient temperature corals (top) and heat-stressed corals (bottom). In all timepoints but T0 ambient temperature, the treatments were found to be significantly different. Sample dispersion was not significant at any of the three timepoints (T0: *PERMDISP*: p = 0.393, T1: *PERMDISP*: p = 0.791, T2: *PERMDISP*: p = 0.732). There was greater separation between communities based on bacteria treatment at T1 and T2 then there was at T0. Pairwise comparisons revealed significant differences between all treatments in heat-stressed corals at T1 and T2. Circles = 95% confidence intervals around bacteria treatments.



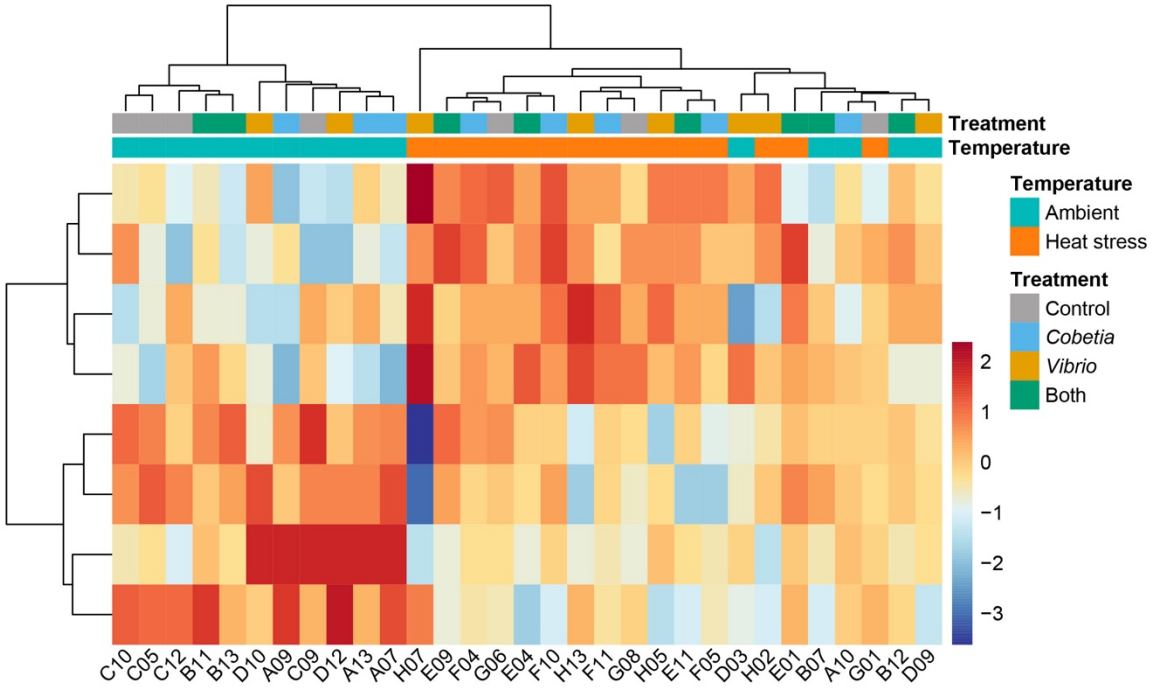
Extended Data Fig. 4. Separation of genome-wide DNA methylation in samples based on timepoint in ambient temperature corals and heat-stressed corals via nMDS plots using Bray-Curtis dissimilarity and hierarchical clustering using the ward.D2 method. The DNA methylation profiles were significantly different between the timepoints in the heat-stressed corals (T0 - T1: R^2 = 0.121, F = 3.720, p = 0.003; T0 - T2: R^2 = 0.071, F = 2.131, p = 0.003; T1 - T2: R^2 = 0.053, F = 1.526, p = 0.012). Circles in nMDS plots = 95% confidence intervals around timepoints.



Extended Data Fig. 5. Percent methylation levels of genes significantly driven by temperature conditions (ambient vs. heat stress) at sampling times T1 (left) and T2 (right) as determined by GLMs. Hierarchical clustering was performed using Euclidean distances and the "ward.D2" agglomeration method. Colors represent the z-score for any given gene. All enriched GO terms due to differences in temperature regimes at T1 and T2 are in Supplementary Table 6a and 6b, respectively.



Extended Data Fig. 6. Genomic context of methylation across genomic regions (a) and along genes (b). Data from all samples (n = 91) were pooled to find where DNA methylation is observed along the *Pocillopora verrucosa* genome. DNA methylation was detected more in genic regions, with higher frequencies of DNA methylation occurring in exons.



Extended Data Fig. 7. Percent methylation levels of glutamatergic synapse genes significantly driven by temperature conditions (ambient vs. heat stress) at T2 (8 genes total) as determined by GLMs. Hierarchical clustering was performed using Euclidean distances and the “ward.D2” agglomeration method. Colors represent the z-score for any given gene.

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